THE REFINEMENT AND CONCENTRATION OF ANTITOXINS *

P. G. Heinemann

From the Department of Hygiene and Bacteriology of the University of Chicago, and the Serum Division of the Memorial Institute for Infectious Diseases, Chicago

The first practical method for the concentration and refinement of diphtheria antitoxin was devised by Gibson.[1] The principle involved in the process was based on experiments carried on by a number of investigators, whose results were on the whole remarkably harmonious. A discussion of earlier work is contained in Gibson's paper. It is generally agreed that the antitoxin in horse blood is contained in the pseudoglobulin fraction.

By the Gibson method the globulins are precipitated by half saturation with ammonium sulfate and the albumins eliminated with the filtrate. Further separation of the globulins is accomplished by dissolving the ammonium-sulfate precipitate in saturated sodium-chlorid solution, and precipitating the antitoxin globulins from this solution with acetic acid. The final precipitate is gathered on hardened filter paper and pressed out, to remove ammonium-sulfate solution as far as possible, then dialysed against running water, and neutralized with sodium carbonate.

Banzhaf later developed a somewhat different method,[2] altho it also was based on the separation of the blood proteins and retention of the pseudoglobulins. The process is as follows:

Citrated plasma is heated to 57 C. for from 12 to 15 hours, and after being cooled, is diluted with half its volume of water. The diluted plasma is mixed with saturated ammonium-sulfate solution in the proportion of 3 parts ammonium-sulfate solution to 7 parts of diluted plasma. The precipitate is gathered on filters and treated in accordance with Gibson's method. The filtrate is mixed with enough saturated ammonium-sulfate solution to bring the saturation to 54%. This second precipitate is gathered on hardened filters, and pressed and dialysed. Neutralization is not necessary. By this method 2 sera are obtained, one of relatively high potency, the other of low.

In 1912 Banzhaf published another paper[3] in which a modification of the previous process is given.

* Received for publication April 10, 1916.

[1] Jour. Biol. Chem., 1906, 1, p. 161.

[2] Collected Studies from the Research Laboratory, Department of Health, City of New York, 1908-9, 4, p. 230.

[3] Ibid., 1912-13, 7, p. 114.

Citrated plasma is diluted with half its volume of water and enough saturated ammonium-sulfate solution added to make a 30% saturation. The mixture is heated to 60 C. in a water bath, and filtered while hot. The precipitate remaining on the filters is either washed with saturated ammonium-sulfate solution diluted with twice its volume of water, or dissolved in water and ammonium-sulfate solution added to bring the saturation to 33⅓%. This solution is filtered, the filtrate united with the first filtrate, and enough saturated ammonium-sulfate solution added to bring the saturation to 50%. The resulting precipitate is pressed and dialysed as in the previous method. Only one serum is obtained by this process.

In a report presented by the Committee on Standard Preparation of Diphtheria Antitoxin to the American Public Health Association in September, 1915, the last method of Banzhaf was described with but one modification. This consists of holding the heated plasma-ammonium-sulfate mixture at 60 C. for 15 minutes before filtering.

There are 2 chief economic advantages accruing from the concentration of antitoxin; (1) Blood can be drawn into a solution of some citrate or oxalate and (2) plasma of relatively low value can be utilized. When the blood is drawn into citrate or oxalate solution, coagulation is prevented, the corpuscles settle, and a yield of plasma is obtained which is larger by as much as 50%, than the amount of serum obtained by spontaneous coagulation. The additional expense incurred in refining processes is more than compensated for in this economy.

Other advantages are the somewhat greater stability of the final product, and the fact that a smaller quantity of serum is required for injection, a three- to four-fold concentration being readily obtained The stability of the product is actually increased, but after some time an opalescence, turbidity, or even a heavy precipitate may form making the serum unsightly, altho its therapeutic value remains unimpaired. Nevertheless physicians frequently object to a turbid serum and manufacturers have found it expedient to warn physicians that turbidity and sediment do not affect the potency of the serum.

If proteins are precipitated in a finished product without reducing the potency, it is clear that there is a possibility of splitting off some protein during the process of refinement without reducing the antitoxic value. Furthermore, the hope that the amount of serum disease following injections of antitoxin would be reduced by using the refined product has been realized in a measure only. On the assumption that serum disease is an anaphylactic reaction it is to be expected that the smaller the number of proteins in a serum, the less the chance of an anaphylactic reaction. Therefore, if the antitoxin globulins can be freed from all traces of other proteins, a high rate of concentration, and a globulin of such purity should be obtained as would greatly

diminish serum disease. The refined products on the market at present are not purified to a degree sufficient to eliminate all nonantitoxic proteins.

Spronck in 1898[4] suggested that the heating of antitoxin to 58 C. for 20 minutes would obviate urticaria. Heating alters some protein, rendering it more precipitable, so that with the elimination of this protein the chances of serum disease are reduced. Heating to 60 C. is also destructive of most bacterial toxins that might accidentally exist in the serum. The aging of serum likewise causes changes in the protein so that serum disease follows its injection less commonly.

It occurred to me that by a combination of methods it might be possible to purify antitoxic serum to a greater degree than heretofore, and during the past year I have succeeded in working out a process which has consistently produced exceptionally high rates of concentration. At the same time the final product is attractive in appearance, green or bluish-green in color, less viscid than some other sera, and water-clear. After 11 months in a syringe one of the first products was still as clear as it was immediately after filtration, altho the color had changed somewhat, the green having passed into a yellowish hue. The quality of the green seems to vary in different sera, but I have observed that with the best results the intensity of the green is pronounced, and I am led to believe that the green color is characteristic of pure globulin solutions.

The process consists of a combination of well-known methods and persistent repetition of certain details until the desired result is obtained. The results are dependable, but in order to obtain uniform products experience and judgment are necessary. The process is briefly as follows:

The first steps are in accordance with Banzhaf's latest method. The citrate or oxalate plasma is diluted with half its volume of water and saturated ammonium-sulfate added in the proportion of 3 parts solution to 7 parts diluted plasma. The mixture is slowly heated in a water bath to 60 C. and held at this temperature for at least 30 minutes. It is then filtered while hot through soft paper filters. After filtration has ceased, the filters with the precipitate collected on them are placed in a suitable vessel, and covered with a measured amount of water. By frequent stirring a fairly homogeneous thick fluid is obtained. To this fluid is added an amount of saturated ammonium-sulfate solution equal to one half the volume of water used. After another thorough stirring the mixture is placed on soft filters and the filtrate mixed with the first filtrate. Finally the filters are filled twice with saturated ammonium-sulfate solution diluted with twice its volume of water. The filtrate contains the last remnants of antitoxin and may be mixed with the previous filtrates. To the

4 Ann. de l'Inst. Pasteur. 1898, 12, p. 697.

mixed filtrates enough saturated ammonium-sulfate solution is added to bring the saturation to 50%.

It is not necessary to mix the different filtrates. Each one can be precipitated separately to advantage. Since the last filtrates are more highly diluted than the first one they will aid in washing remnants of nonantitoxic proteins from the precipitate which is gathering on the filters. This precipitate is yellowish or yellowish-brown and the filtrate is of similar color. After filtration has ceased, the filters with the precipitates are stirred in a measured amount of water; after solution the fluid is strained through cheese cloth and the remaining paper pulp pressed out. The pressed pulp is again mixed with a measured amount of water, strained and pressed, and this process repeated a third time. All globulins are washed out of the paper by this method.

The strained fluids are mixed and a volume of saturated ammonium-sulfate solution equal to the total amount of water used for solution is added. The precipitate is gathered again on soft filters. The precipitate and filtrate are now much lighter in color than after the first precipitation.

The precipitate should be dissolved and precipitated a second, or even a third, time until the precipitate is bluish-green and the filtrate colorless. Nonantitoxic proteins are present as long as the filtrate is colored yellow or yellowish-brown. The precipitate can now be pressed out and dialysed. In most cases better results are obtained when heat is applied a second time. To this end the precipitate is dissolved in a measured amount of water, the paper pulp pressed out as before and the strained fluid measured. The fluid is of course of a larger volume than the amount of water added for solution, because of the volume occupied by the precipitate and the presence of some 50% ammonium-sulfate solution in the moist precipitate. It may be assumed that the difference in volume is half the saturated ammonium-sulfate solution, altho it is really somewhat short of the assumed amount, and on this basis enough saturated ammonium-sulfate solution is added to make a 30% saturation. A very slight precipitate will form if enough ammonium sulfate is present. If this precipitate does not appear more ammonium-sulfate solution should be added until a slight cloudiness is produced. This mixture is then heated to 60 C. again, and held at this temperature for 15 minutes. It is then filtered while hot, and the precipitate washed out as before. The mixed filtrates are brought to a 50% saturation with ammonium-sulfate solution and the precipitate gathered on hard filters. The color of the precipitate is now bluish-green, and after the ammonium-sulfate solution has been pressed out, dialysis can be commenced. The pressed precipitate is intensely bluish-green, resembling a substance containing copper. By the second heating a precipitate is formed which is not soluble in 30% ammonium-sulfate solution and which perhaps consists of a globulin which has been split off.

The resulting globulin solution is generally satisfactory. It may not be quite water-clear, but is green and passes readily through a Berkefeld filter, as it is not very viscous. By this method I have obtained sera of 800, 900, and in one instance 1000 units from plasma which tested less than 200 units, and probably but little more than 100 units.

Frequently the serum at this stage is turbid. This seems to occur chiefly when the blood is obtained from old horses or from a horse

that has been bled to death. A serum of this kind can be clarified completely by the following method:

It is diluted with twice its volume of water and enough saturated ammonium-sulfate solution to make a 7 to 3 saturation is added. This mixture is heated to 60 C. and filtered while hot. The precipitate which remains on the filters is dissolved in water and saturated ammonium-sulfate added to make a one-third saturation. This mixture is filtered and the filtrates united as in the beginning of the process. The globulins are precipitated by addition of saturated ammonium-sulfate solution to a 50% saturation. The precipitate when gathered on the hard filters is intensely bluish-green and after dialysis the solution is always perfectly clear. There is a loss of about one-third of the volume and a corresponding increase in potency. By this method of double concentration I have obtained sera of from 1,200 to 1,400 units' potency from plasma testing less than 200 units, and in one instance a 2,000 unit serum from plasma testing less than 300 units.

The process seems of course rather laborious. Most of the work, however, can be done by inexpensive helpers. The expense of ammonium sulfate is somewhat greater than in the case of the former method, but this expense can be reduced by using the last colorless filtrate over again as containing a 50% saturated ammonium-sulfate solution. It can be used whenever a 30 or 33% saturation is required. A large number of soft filters are used, but relatively few hard filters are required. The cost of filters is therefore not greatly increased since hard filters are much more expensive than soft ones. It is not essential to heat the globulin solution more than once during the first concentration if a second concentration is to be carried out.

The advantages gained appear quite sufficient to offset the extra expense, for a relatively high potency serum with 18 to 20% solids can be obtained from a plasma which otherwise would be valueless, and the stability of the serum is also of considerable economic value.

Sera prepared by the described method have been used in the Durand Hospital of the Memorial Institute for Infectious Diseases in Chicago. Dr. George H. Weaver has made the following observations on the occurrence of serum disease after injections of a serum prepared by the new process as compared with serum prepared by the Banzhaf method: "Serum No. 446, concentrated according to Banzhaf's method, was used in 21 cases. Reactions were observed in 10 cases, or 47.6 percent. Of these reactions two were severe, four moderate, and four mild. Serum No. 519, concentrated according to Banzhaf's method, was used in 31 cases. Reactions were observed in 12 cases, or 38.7 percent. Of these seven were severe, two moderate and three mild. Serum No. 543, concentrated according to the new method, was

used in 40 cases. There were but three reactions, or 7.5 percent. Of these one was severe and two moderate. The serum was used in a number of additional cases, but since these had been treated with antitoxin before being taken to the hospital they had to be excluded."

There is little doubt about the feasibility of concentratnig tetanus antitoxin according to the same principle. I have myself concentrated hog-cholera serum successfully. The serum used was sent to me for experimental purposes by Dr. C. H. Stange of Iowa State College at Ames. The final product was sterile, and water-clear with a slightly pinkish color due to remnants of hemoglobin which were impossible to exclude, tho if hog's blood were drawn into a citrate or oxalate solution a perfect product could be obtained. In this case 7 c.c. of the refined product protected, while of the original serum from 20 to 30 c.c. were required. This work bears out a statement made by Eberson[5] that the antibodies of hog-cholera serum are contained in the pseudoglobulin fraction of hog's blood.

The work reported in this paper demonstrates 2 facts: first, antitoxic sera concentrated by the Banzhaf method contain, besides pseudoglobulins, varying amounts of other blood proteins which are not readily eliminated. What these proteins are I have not determined, but the results of my work suggest that precipitation of protein in serum when it ages is due to the presence of nonantitoxic proteins and not to true antitoxic globulins. Moreover, there is support for the belief that serum disease is largely due to the presence of these nonantitoxic proteins. Therefore, the amount of serum disease is reduced in proportion to the completeness of the elimination of nonantitoxic proteins.

Second, it has been demonstrated that pseudoglobulins may be split into fractions and that some of these do not contain antitoxin while others contain it in highly concentrated form. The possibility suggests itself that by repeated heating of antitoxic serum with 30% ammonium-sulfate solution the antitoxin may be condensed into a very small fraction of pseudoglobulin. If this were successful considerable light might be thrown on the condition in which antibodies exist in the blood. The high rate of concentration resulting from my method is due of course simply to the elimination of nonantitoxic proteins, which otherwise would require a greater amount of water for solution, unless the solids in the final product are excessively high.

[5] Jour. Infect. Dis., 1915, 17, p. 339.

CONCLUSION

A serum of attractive appearance can be produced. It is water-clear for at least 1 year, probably for a much longer period. No turbidity develops, nor does a sediment form during this time.

A higher concentration can be obtained than by former methods, because nonantitoxic proteins are eliminated. The solids of the product obtained by the new method need not be higher than from 18 to 20%.

The consistency of the serum enables it to flow readily through Berkefeld filters, a pressure of from 15 to 20 pounds being usually sufficient to maintain a constant flow when "v" or "n" density filters are used. Furthermore, it is easily discharged through a hypodermic needle.

The antitoxic globulins realized by the new method are easily soluble and dialysis is consequently rapid.

Plasma of 100 and 200 units can be used to advantage, thus being offset the additional expense of production.

Nonantitoxic proteins are eliminated in large measure and serum disease, as far as present observations go, is greatly reduced.